Role of Fluorescent Pseudomonads and Their Pectolytic Enzymes in Spoilage of Fresh and Fresh-Cut Produce

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[1] Mention of trade names or commercial products in this chapter is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

16.1 INTRODUCTION

Fresh and fresh-cut produce have become the fastest growing food category in the supermarket during the last two decades. A recent USDA survey [1] showed that the demand for fresh produce increased by more than 12% in the last decade and per capita consumption jumped from 284 pounds in 1987 to 318 pounds in 1997. To meet the market expansion, new strategies are required to improve the production of these commodities on the farm and to reduce the losses caused by physical, physiological, and microbiological disorders after harvest. An estimated 10 to 30% of fresh fruits and vegetables produced in the U.S. are wasted after they are harvested [2]. A large part of these losses are due to spoilage caused by bacteria, fungi, or yeasts [3–6]. The spoilage of acidic fruits such as apples, oranges, and strawberries is usually caused by molds, yeasts, or lactic acid bacteria (LAB) [5,6]. However, the spoilage of fresh produce with neutral pH, including edible roots/tubers and salad vegetables, is often the result of pectolytic bacteria causing a form of "soft rot" [3,4]. Results from a series of USDA surveys show that "bacterial soft rot" accounts for a very large proportion of postharvest disorders in potato, tomato, lettuce, bell pepper, and cucumber shipments at wholesale produce markets in New York [7–9]. Apart from the economic impact, soft-rotted produce more often harbors human pathogens such as *Salmonella* than their healthy counterparts [10] and has become an important food safety concern [11].

Bacterial soft rot is commonly known to be caused by three groups of *Erwinia,* including *E. carotovora* subsp. *carotovora* (Ecc), *E. carotovora* subsp. *atroseptica* (Eca), and *E. chrysanthemi* (Ech). However, pectolytic bacteria in at least six other genera including *Pseudomonas, Xanthomonas, Cytophaga, Flavobacterium, Bacillus,* and *Clostridium* can be involved [4,5]. A series of studies conducted in our laboratory during the 1980s [12–14] showed that over 40% of the rotted fruits and vegetables collected at retail and wholesale markets were likely caused by non-*Erwinia* soft-rotting bacteria including *Cytophaga*, *Xanthomonas*, and pectolytic fluorescent (PF) pseudomonads. Bartz [15] also found that PF pseudomonads

accounted for almost one-third of the soft-rotting bacteria isolated from decayed tomatoes. The assumption that PF pseudomonads consisting of *P. fluorescens* and *P. viridiflava* are more likely to be involved in spoilage of refrigerated produce than other pectolytic bacteria can be attributed to at least two reasons. First of all, these pseudomonads are nutritionally versatile and capable of growing in a simple salt solution containing only four minerals and one of several utilizable carbon sources [14]. Second, both *P. fluorescens* and *P. viridiflava* are psychotropic and capable of inducing soft rot of fresh produce that is stored at 10°C or below [14,16,17]. Other genera of soft-rot bacteria usually grow very poorly and are unable to induce tissue maceration at low temperatures. Possibly because of these mentioned capabilities, fluorescent pseudomonads, including pectolytic strains, are commonly found on the surfaces and often constitute a major component of native microflora on fresh and minimally processed produce [16–25].

The soft-rot symptoms caused by PF pseudomonads are in general similar to those caused by Ecc, Eca, and Ech. However, under the most favorable conditions PF pseudomonads are less virulent than the erwinias. It is now generally believed that the ability of soft-rot erwinias and pseudomonads to macerate plant tissue results mainly from their ability to degrade plant cell walls by producing an array of pectin-degrading enzymes [26]. The enzymatic and molecular mechanism by which pectolytic erwinias cause soft-rot disease in plants has been extensively investigated and reviewed [26–29]. However, the role of PF pseudomonads and their pectic enzymes in spoilage of fresh and fresh-cut produce has not yet been studied to the same extent as the soft-rot erwinia systems. The subjects to be discussed in this chapter include: (1) the distribution and relationship of PF pseudomonads to spoilage of fresh produce; (2) pectic and other depolymerizing enzymes produced by PF pseudomonads; (3) biochemical and molecular genetic evidence that a single alkaline pectate lyase (PL) is required for induction of soft rot, (4) interactions between fluorescent pseudomonads, native microflora, and human pathogens (*Listeria monocytogens, Escherichia coli* O157:H7, and *Salmonella*) on fresh produce; and (5) potential postharvest treatments for inactivation of unwanted microorganisms on fresh produce.

16.2 PF PSEUDOMONADS AS A MAJOR CAUSE OF PRODUCE SPOILAGE

16.2.1 Physiological Diversity of PF Pseudomonads

PF pseudomonads represent a very heterogeneous taxonomic group mainly consisting of *P. viridiflava* [30] and five biovars of *P. fluorescens* [31]. Soft-rotting strains of *P. fluorescens*, often referred to as *P. marginalis* in the plant pathology literature [32], were the first among PF pseudomonads to be recognized as a soft-rotting pathogen of head lettuce in the field and after harvest [33,34]. With the exception of this disease-causing ability, *P. marginalis* is indistinguishable from other strains of *P. fluorescens* genetically and physiologically [31,33]. Until now, the description

of *P. marginalis* continues to be used for those soft-rotting pseudomonads that are fluorescent and positive in oxidase and arginine dehydrogenase activities [32,34]. Although the type strains of *P. marginalis* previously available from culture collections were identified as *P. fluorescens* biovar II [31], soft-rotting strains belonging to the four other biovars of *P. fluorescens* have been isolated. Following the characterization of 55 strains of PF pseudomonads isolated from naturally rotted specimens, Liao and Wells [14] found that only 19 of those strains exhibited the characteristics typical of biovar II. The remaining 36 strains were identified as *P. fluorescens* biovar IV (9 strains), *P. fluorescens* biovar V (11 strains), and *P. viridiflava* (16 strains). Brocklehurst and Lund [16] reported that *P. fluorescens* strains belonging to biovars I and III were possibly involved in the spoilage of cabbage stored in the cold. Diverse biovars of *P. fluorescens* have also been shown to be associated with spoilage of endive leaves and ready-to-use salad vegetables [23,35]. These reports and others not cited suggest that PF pseudomonads are likely responsible for a substantial proportion of postharvest rot of fresh and fresh-cut produce that is stored at low temperatures.

It should be noted, however, that other species of fluorescent pseudomonads including *P. aeruginosa* [36] and *P. cichorii* [7] have also been reported to be associated with spoilage of potato tubers and leafy vegetables. To the best of our knowledge, neither *P. aeruginosa* nor *P. cichorii* has ever been shown to produce pectolytic enzymes required for induction of soft rot as discussed below. In contrast, certain *P. syringae* pathovars including pv. *lachrylmans,* although exhibiting strong pectolytic activity *in vitro,* are unable to cause soft rot on potato tuber slices or cucumber fruits [33]. *P. syringae* pathovars are very closely related to *P. viridiflava* genetically and physiologically. So far, there is no report showing the involvement of pectolytic *P. syringae* in spoilage of fresh produce. Therefore, PF pseudomonads to be discussed in this chapter are limited to the soft-rotting strains of fluorescent pseudomonads, mainly consisting of *P. fluorescens* and *P. viridiflava.*

16.2.2 Occurrence of PF Pseudomonads on Fresh Produce

Unlike soft-rot erwinias, PF pseudomonads are generally considered weak and opportunistic pathogens that do not cause large-scale disease outbreaks in the field. However, these bacteria are widespread in nature and can be isolated from very diverse environments and plant sources including soil, water, root rhizospheres, and surfaces of fruit and vegetables. As common epiphytes, they can become a major component (up to 40%) of the native microflora on potato tubers [37,38], collards [39], peas [40], tomatoes [41], spinach [42], lettuce [43–45], cabbage [46], and salad vegetables [47–49]. Although the direct involvement of these pseudomonads in spoilage is difficult to demonstrate, it is generally assumed that reduction in shelf life of fresh produce as a result of spoilage may be caused by complex interactions between PF pseudomonads and nonpathogenic microflora including lactic acid bacteria (LAB), yeasts, or fungi. Because of their ubiquity and potential to induce tissue maceration at low temperatures, PF pseudomonads are expected to play a very critical role in the quality and safety of fresh produce.

16.3 BIOCHEMICAL CHARACTERIZATION OF PECTATE LYASE

16.3.1 PRODUCTION OF PECTIC ENZYMES AND OTHER DEPOLYMERASES BY PF PSEUDOMONADS

PF pseudomonads produce at least four types of pectinases: polygalacturonase (PG), pectate lyase (PL), pectin lyase (PNL), and pectin methyl esterase (PME). PG is a glycosidase that cleaves α-1,4 glycosidic bonds between uronic acid residues in polygalacturonic acid (PGA) by hydrolysis. PL is a lytic enzyme that cleaves α-1,4 glycosidic bonds between uronic acid residues in PGA or low-methylated pectin by *trans*-elimination. PNL is also a lytic enzyme that cleaves uronic acid residues in highly (> 91%) methylated pectin. PME is a saponifying enzyme that causes the hydrolysis of methyl ester groups in highly methylated pectin with the production of methanol and PGA. The method for analyzing the activity of each enzyme [50–54] has been developed mainly based on detection of unsaturated oligogalacturonate products generated by PL or PNL or detection of saturated digalacturonates or methanol products generated, respectively, by PG and PME. Based on the data obtained so far, production of PG and PME by PF pseudomonads appears to be rare and has been demonstrated only in a few strains of *P. fluorescens* so far examined [53,54]. Production of PNL was detected only in cultures of certain *P. fluorescens* strains that had been exposed to DNA-damaging agents such as mitomycin C, UV irradiation, and nalidixic acid [55,56]. As will be discussed in more detail, almost all of the PF pseudomonads so far examined produce PL in culture either constitutively or inducibly. Production of PL therefore represents a common feature among soft-rotting pseudomonads, suggesting that PL may be the primary enzyme required for induction of soft rot [57]. On the contrary, production of PME, PNL, and PG appears not to be essential for induction of soft rot but may aid the survival and growth of these pseudomonads in plants or other environments.

In addition to pectin-degrading enzymes, PF pseudomonads also produce proteases (Prt) [58] and cellulases (Cel) [59], but not lipases and amylases [60]. Production of lipases, however, has been detected in at least two *P. fluorescens* strains used for biological control [61]. Production of Prt and Cel by pseudomonads is possibly for the purpose of catabolic or nutritional functions. Both enzymes degrade polymeric substrates (protein or cellulose) readily available in host plants or environments into monomeric end products that can be utilized by bacteria as energy or carbon sources. When inoculated onto plants, purified Prt or Cel is unable to cause visible disintegration of plant tissues. It has yet to be determined, however, whether the combined action of PL and other depolymerases such as Prt, Cel, or lipase may augment the extent of spoilage or tissue maceration. Although the biological functions of all the diverse extracellular enzymes produced by PF pseudomonads are not clear, Prt produced by *P. fluorescens* has been extensively investigated. Analysis of the concentrated culture supernatant of *P. fluorescens* (CY091) by isoelectric focusing (IEF) electrophoresis and overlay enzyme-activity staining revealed the presence of at least two Prts [62]. Two biocontrol strains (B52 and A506) of *P. fluorescens*

have also been shown to produce more than one Prt [61; J. Loper, personal communication]. The predominant Prt produced by *P. fluorescens* CY091, designated AprX, has been characterized and the gene operon (in 7.3-kb genomic fragment) encoding the structural enzyme protein and its secretory apparatus has been cloned and sequenced [58]. The Prt AprX has an estimated molecular mass of 50 KDa and is a zinc-metalloprotease requiring Ca^{+2} for activity. Production and secretion of AprX by strain CY091 is dependent on Ca^{+2} or Sr^{+2}, and two conserved sequence domains associated with Ca^{+2} or Sr^{+2} binding have been identified. As an extracellular alkaline enzyme, AprX exhibits 50 to 60% identity in amino acid sequence to related proteases produced by *P. aeruginosa* [63] and *E. chrysanthemi* [64].

PF pseudomonads can also produce other types of depolymerases or secondary metabolites to enhance their ecological fitness or pathogenesis requirement. Production of a peptidolipid biosurfactant, viscosin, by a pectolytic strain of *P. fluorescens* has been shown to facilitate the initiation and spread of soft rot [65]. Although the health benefit of a diet rich in fresh produce is well known, fruits and vegetables have been shown to contain a potentially hazardous compound named "rutin" [66]. Rutin is a flavonol glycoside consisting of the mutagenic aglycone quercetin and the disaccharide rutinoside. Certain PF pseudomonads are able to produce a glycosidase to degrade rutin and thereby minimize the safety hazard associated with this compound [67].

16.3.2 Description of the *Erwinia* Pectic Enzyme System

Much of our knowledge about the enzymatic mechanism of soft-rot pathogenesis was derived from studies with *Erwinia*. Soft-rotting *Erwinia* are well known for their ability to produce a wide variety of pectic enzymes including PL, PNL, PME, PG, and PAE (pectin acetylesterase). Each pectinase cleaves a preferred substrate (pectin or pectate) by hydrolysis, *trans*-elimination, or saponification. Although the pathological function of each pectinase is not fully understood, PL is generally believed to be the principal enzyme involved in tissue maceration, electrolyte loss, and cell death [68]. PLs produced by *Erwinia* species are unique for their occurrence as multiple (greater than five) isozymes with isoelectric points (pIs) ranging from 4.0 to 10.0 [69]. It has been demonstrated that the alkaline PL (pI > 9.0) is more efficient than neutral or acidic PLs in inducing tissue maceration [70], and that an alkaline PL by itself is sufficient to cause tissue maceration even in the absence of live bacteria [71]. The biochemical basis for the difference in tissue-macerating ability among PL isozymes has not yet been determined. In addition, the pathological basis for producing more PLs than are required for induction of soft rot by *Erwinia* is not fully understood. Whether production of multiple PL isozymes is required for attacking different host plant species or different organs within the same species needs to be further investigated [72]. Because of their pathological and biotechnological importance, the molecular mechanisms by which soft-rot *Erwinia* mediate the synthesis and secretion of various depolymerases possibly in response to environmental changes or stresses have been extensively investigated and reviewed [27–29].

16.3.3 Description of the *Pseudomonas* Pectic Enzyme System

Unlike the complex range of pectinases produced by soft-rot erwinias, the pectic enzyme system of PF pseudomonads is much simpler. So far, only a few strains of *P. fluorescens* and *P. viridiflava* have been shown to produce PME, PG, and PNL. However, all but one strain of PF pseudomonads examined in our laboratory produce PL [57]. In order to determine whether PF pseudomonads also produce multiple PL isozymes, the IEF gel electrophoresis and overlay enzyme-activity staining techniques [69] have been applied to analyze the IEF profile of PLs produced by 18 strains of PF pseudomonads. All 8 strains of *P. viridiflava* and all 10 strains of *P. fluorescens* investigated in our laboratory produce a single PL with an approximate pI of 9.7 to 10.0 [57]. The IEF-overlay enzyme-activity staining techniques have also been used to analyze the IEF profiles of PLs produced by other non-*Erwinia* pectolytic bacteria. Results obtained so far suggest that production of a single alkaline PL is a common feature among non-*Erwinia* pectolytic bacteria including *Cytophaga johnsonae* [57,73], *Xanthomonas campestris* [74,75], *Bacillus subtilis* [76–79], *Clostridium* spp. [80,81], and possibly *E. rubrifaciens* [82].

16.3.4 Purification, Enzymatic Properties, and Tissue-Macerating Ability of *Pseudomonas* PLs

Because of the simplicity of the pectic enzyme system, PLs produced by non-*Erwinia* soft-rotting bacteria including *P. fluorescens*, *P. viridiflava*, *C. johnsonae*, and *X. campestris* can be easily purified from culture filtrates by two simple steps including ammonium sulfate precipitation and anion-exchange chromatography [57]. Analysis of PL samples by SDS-polyacrylamide gel electrophoresis showed that the enzymes had been purified to near homogeneity following the two purification steps. Molecular weights (M_r) of PLs from *P. fluorescens* (CY091) and *P. viridiflava* (SF312) were estimated to be 41 and 42 KDa, respectively, based on their electrophoretic mobility in SDS-polyacrylamide gels. Further analysis of purified PLs by IEF gel electrophoresis confirmed the alkaline nature of the enzymes (pI = 9.7 to 10). However, *Pseudomonas* PLs appear to migrate in SDS-polyacrylamide gels at a rate slightly slower than expected for the sizes of proteins predicted from the genes cloned [83–85], possibly due to the unique β-helix protein structure similar to that demonstrated for *E. chrysanthemi* PLc [86]. In addition to a slight difference in M_r and pI, the PLs from *P. fluorescens* and *P. viridiflava* can be distinguished by differences in other biochemical properties including Km, Vmax, and optimal pH and temperature for activity [87]. For both PLs, the optimal Ca^{+2} concentration for activity is 0.5 mmol/L, the optimal pH for activity is 8.5 to 9.0, and they are stable at low temperatures (25°C or below) for at least 30 d. However, at 37°C, the activity decreased 50% in 36 h. Thermostability of both enzymes at elevated temperatures (48°C or higher) increases in the presence of $CaCl_2$ or a positively charged molecule such as polylysine and decreases in the presence of a negatively charged molecule such as heparin. Both PLs exhibit differential degrees of sensitivity to group-specific inhibitors such as iodoacetic acid and diethylpyrocarbonate, indicating that sulfhydryl

and imidazole groups are important for their catalytic function [87]. PLs purified from culture supernatants of *C. johnsonae* and one strain each of *P. fluorescens* and *P. viridiflava* were all able to induce soft rot on potato tuber slices to different degrees. In general, PLs from the two pseudomonads are about 10-fold more efficient in inducing tissue maceration than the PL from *C. johnsonae* [57]. When inoculated onto potato tuber slices, a minute amount (less than one unit of activity; one unit of activity being the amount of enzyme required to release 1 μmol of unsaturated uronides) of purified PL from either pseudomonad is sufficient to induce maceration of plant tissue even in the absence of live bacteria [57,87].

16.4 MOLECULAR GENETIC ANALYSIS OF PL PRODUCTION BY PF PSEUDOMONADS

16.4.1 ANALYSIS OF TRANSPOSON (TN*5*) MUTANTS DEFICIENT IN PL PRODUCTION AND SECRETION

Two classes of *P. viridiflava* and *P. fluorescens* mutants defective in pectolytic activity and designated as Pel$^-$ and Rep$^-$, respectively, have been isolated using transposon Tn*5* mutagenesis [71]. The Pel$^-$ mutation resulted from the insertion of Tn*5* into the structural *pel* gene, whereas Rep$^-$ mutation resulted from the insertion of Tn*5* into one of two regulatory genes, designated *repA* (= *gacS* = *lemA*) and *repB* (= *gacA*). The Rep$^-$ mutants exhibit pleotrophic phenotypic changes including the loss of the ability to synthesize PL, Prt, exopolysaccharides, and fluorescent siderophores. Since the loss of pectolytic activity in Pel$^-$ and Rep$^-$ mutants was always accompanied by the loss of the soft-rotting ability on bell pepper fruits [88–90], production of PL is absolutely required for soft rot development. However, as discussed above, production of additional depolymerases such as Prt is not essential for induction of soft rot. Prt$^-$ mutants resulting from the transposition of Tn*5* into the structural *aprX* gene retain the wild-type level of tissue-macerating ability of *P. fluorescens* [89] and *P. viridiflava* [90]. Production of Prt does not appear to play a significant role in induction of soft rot.

16.4.2 CLONING AND CHARACTERIZATION OF THE *PEL* GENES FROM NON-*ERWINIA* PECTOLYTIC BACTERIA

Current knowledge about *pel* genes was derived primarily from the studies of soft-rotting *Erwinia*. A number of reviews on this subject are available in the literature (for examples, see 26–28,72). Recently, *pel* genes have been cloned from several non-*Erwinia* phytopathogens including *P. viridiflava* [84], *P. fluorescens* [85], *P. marginalis* [91], *P. s.* pv. *lachrymans* [92], *Bacillus* spp. [93–95], *X. c.* pv. *campestris* [74], *X. c.* pv. *vesicatoria* [75], and *X. c.* pv. *malvacearum* [83]. Nucleotide sequences of *pel* genes cloned from these bacteria have been determined and found to be closely related to alkaline PLs (PLd and PLe) of *E. chrysanthemi* [83]. These *pel* genes usually encode pre-Pel proteins consisting of 377 to 380 amino acid (a.a.) residues with a signal peptide consisting of 26 to 29 a.a. at the N-terminus. Four conserved sequence domains presumably involving Ca^{+2} binding, catalytic activities, and protein-export functions

were revealed. Multiple sequence alignment analysis shows that PL proteins of non-*Erwinia* phytopathogens including *Xanthomonas*, *Pseudomonas*, and *Bacillus* constitute a distinct cluster that shows 20 to 43% a.a. identity to the four established PL enzyme families of *Erwinia* [83]. Two lines of evidence further confirm the alkaline PL produced by non-*Erwinia* soft-rotters as the single factor responsible for tissue maceration. *Escherichia coli* cells carrying a *Pseudomonas pel* gene were able to induce tissue maceration inside potato tubers and under anaerobic conditions [85]. Restoration of soft-rotting ability in Pel$^-$ mutants could be accomplished by transferring the functional *pel* gene into the mutants [84]. These results further confirm the earlier conclusion that non-*Erwinia* soft-rotting bacteria including PF pseudomonads produce a single PL for induction of soft rot as compared to multiple PLs produced by *Erwinia*.

16.4.3 Mechanisms Regulating the Production and Secretion of PL

Although much is known about the molecular genetic mechanisms by which soft-rot *Erwinia* regulates the production of pectic enzymes [26–28], very little is presently known about the mechanism by which PF pseudomonads mediate the synthesis of PL. Pleotropic mutants of *P. fluroescens* and *P. viridiflava* displaying the simultaneous loss of pectolytic and proteolytic activities have been identified by transposon mutagenesis [88–90]. Results from Southern blot analysis using an internal fragment of Tn*5* as a probe revealed that these mutants were derived from the transposition of Tn*5* into one of two distinct genomic fragments. Two functional genes designated *gacS* (= *repA* or *lemA*) and *gacA* (= *repB*) in these two fragments have been identified, cloned, and confirmed by complementation studies. Following nucleotide sequence analyses, the *gacS* and *gacA* genes were predicted to encode a sensory and a regulator protein, respectively, in a two-component regulatory protein family [96–100]. The *gacS/gacA* pair thus likely acts in concert to mediate the production of PL, Prt, EPS, and siderophores [88–90], possibly in response to environmental needs or stresses. The two-component regulators GacS and GacA in a biological control strain of *P. fluorescens* have been shown to regulate the production of phospholipase C [96], lipase [61], and antibiotics [97–99] in biological control strains of *P. fluorescens*. The GacS/GacA system is also involved in the formation of disease lesions on snap beans by *Pseudomonas syringae* pv. *syringae* [100]. This system also interacts with the stationary-phase factor δ^s (encoded on *rpoS*) playing a predominant role in the regulatory cascade controlling stress responses in a biocontrol strain of *P. fluorescens* [101]. The global activator GacA of *P. aeruginosa* interacts with a quorum-sensing regulatory system (LuxR-LuxI) to control the production of the autoinducer-butyryl-homoserine lactone [102]. It has not yet been investigated whether the RpoS and autoinduction regulatory cascade as demonstrated in other strains of *P. fluorescens* or *P. aeruginosa* also operates in PF pseudomonads to control the production of tissue-macerating factor PL.

A group of *P. viridiflava* mutants failing to excrete PL and Prt across the outer membrane have been generated by transposon mutagenesis [71]. These secretion-defective mutants, designated Out$^-$ mutants, resulting possibly from the insertion of

Tn*5* into a cluster of genes in the Type II secretion gene family [103,104], were unable to induce soft rot on potato tuber slices or bell pepper fruits [71]. The synthesis and secretion of PL thus represent two consecutive functions required by *P. fluorescens* and *P. viridiflava* to be efficient soft-rotting pathogens.

Production of PL in certain strains of *P. fluorescens* is induced by pectic substrates [53,105] or by plant tissue extracts [106–108]. However, in other *P. fluorescens* strains, production of PL is not affected by the type of carbon source included in the medium [53,108]. Recently, we investigated the mode of PL production in 24 strains of *P. fluorescens* and found that production of PL in 4 out of 24 strains was not induced by pectic substrates but by Ca^{+2} [109]. These four strains produce 10 times more PL in medium containing 1 m*M* $CaCl_2$ than in one containing no $CaCl_2$ supplement. Presence of $CaCl_2$ in the medium not only affects the amount but also the final destination of PL. Over 86% of total PL produced by strain CY091 in $CaCl_2$-supplemented medium was excreted into the culture fluid. By comparison, only 13% of total PL produced by this strain in $CaCl_2$-deficient medium was detected in the extracellular fraction. The effect of Ca^{+2} on PL (and also Prt) production is concentration-dependent and can be replaced by Sr^{+2}, but not by Zn^{+2}, Fe^{+2}, Mn^{+2}, Mg^{+2}, or Ba^{+2}. Because of the indispensable role of Ca^{+2} in PL production and pectic degradation, the potential of using ion-chelating agents such as EDTA for control of *Pseudomonas* rot has been investigated [109]. Treatment of potato tuber disks with 40 ppm of EDTA especially in the presence of nisin (a bacteriocin) is effective in suppressing the development of soft rot [109,110].

16.5 INTERACTIONS OF PF PSEUDOMONADS AND NATIVE MICROFLORA ON FRESH PRODUCE

The changes in microflora on fresh and fresh-cut produce as affected by processing, decontamination treatments, and storage conditions have been extensively investigated and reviewed [5,6]. The numbers and the types of microorganisms identified are variable and largely dependent on the sources of the samples analyzed. The population of mesophilic bacteria as determined on plate count agar can range from 10^3 to 10^9 colony-forming units (CFU) per gram of tissue. Very diverse groups of microflora are present on the surfaces of fresh fruits and vegetables. In addition to fluorescent pseudomonads and *Erwinia*, other genera of microflora including *Serratia*, *Klebsiella*, *Citrobacter*, *Enterobacte,* yeast, and LAB have been detected on various types of produce [17,18–20,22,40,42,43,45,47,48]. PF pseudomonads often constitute a major proportion of native flora on salad vegetables [17], shredded lettuce [45], cauliflower florets [47], endive leaves [22], spinach [42], tomatoes [41], and alfalfa seed [25], and appear to play the critical role in the development of soft rot or spoilage. As discussed above, softening and maceration of plant tissues results mainly from the action of PL or other depolymerases produced by pectolytic *Erwinia* and fluorescent pseudomonads.

The coliforms and enterobacteria present on the surfaces of fresh produce are generally considered saprophytic and nonpectolytic, although production of PL and exoPG within the cells has been detected in certain strains of *Klebsiella* and *Yersinia* [115,116]. The role of these enterobacteria in spoilage of fresh produce is unclear,

and a direct correlation between the number of bacteria present and the degree of spoilage observed has not yet been consistently demonstrated. It appears that the shelf life of fresh produce is more dependent on the type of microorganisms present than on the total number of bacteria detected. Pectolytic microflora including *Erwinia* [3–6], PF pseudomonads [3–6,35], LAB [111,112], and yeasts [113,114] are more likely to cause spoilage than nonpectolytic flora such as *Klebsiella* and *Citrobacter*. Under natural conditions, spoilage of fresh and fresh-cut produce results largely from the complex interactions between pectolytic and nonpectolytic microflora present on the surfaces of produce. A nonlinear mathematical model to predict the growth of *P. marginalis* and its relationship to vegetable spoilage has been proposed [117,118]. It should be noted, however, that not all produce spoilage is microbiological in nature. Physiological spoilage of fresh produce can be caused by endogenous pectic enzyme activities or fermentative reactions inside plant tissues [24].

16.6 INTERACTIONS OF PF PSEUDOMONADS AND HUMAN PATHOGENS ON FRESH PRODUCE

The ability of pathogenic pseudomonads to infect and multiply in plants is mainly due to their ability to produce enzymes, toxins, or other virulence factors to disrupt plant cells in order to obtain nutrients for growth [119]. Recently, a number of gastrointestinal human pathogens including *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* have also been found to survive and grow on cut surfaces of fresh fruits or vegetables. It is unclear, however, as to how human pathogens acquire nutrients for growth on uninjured plants. With the exception of *Yersinia enterocolitica*, the other human pathogens including *E. coli*, *Salmonella*, and *L. monocytogenes* do not produce enzymes or toxins associated with pathogenicity on plants. Based on a recent survey [116] conducted in our laboratory, *Yersinia enterocolitica* is the only foodborne pathogen that has been shown to produce pectic enzymes, including one endolytic PL and one exolytic PG. Pectic enzymes produced by *Yersinia* and *E. coli* strains carrying the genes coding for these two enzymes are accumulated within the perplasmic space of bacterial cells and were unable to disrupt plant cells and to induce maceration of potato slices [116]. It is not known whether pectolytic flora including PF pseudomonads interfere with the colonization of plants by human pathogens. The data accumulated so far suggest that the presence of PF pseudomonads on fresh produce can exert either positive or negative effects on the survival and growth of foodborne pathogens, as discussed below.

Wells and Butterfield [10] were the first to report a higher incidence of *Salmonella* contamination on rotted produce than on apparently healthy plant counterparts (18 to 20% compared to 9 to10%, respectively). The higher incidence of *Salmonella* contamination was thought to be caused by enhanced growth of the pathogen in rotted tissue. A challenge study showed a 5- to 10-fold increase in the population of *S. typhimurium* was observed in potato disks coinoculated with *E. carotovora* or *P. viridiflava* [10]. Carlin et al. [22,23] also found that increases in the population of *L. monocytogenes* were directly correlated with the extent of spoilage of fresh endive leaves. The rotted tissue may provide the nutrients needed for the growth of

human pathogens. Furthermore, the macerated tissue may also serve as a source or vehicle for dissemination of foodborne pathogens and spread of disease [120].

Rotted tissues infected with fungal pathogens are also more likely to harbor *Salmonella* than their healthy counterparts [121]. Foodborne pathogens such as *Salmonella* and *L. monocytogenes* usually do not grow, or grow very poorly, on acidic fruits (pH < 4) such as apples or oranges. The growth of postharvest rot pathogens in fruits can markedly change the pH surrounding the infected tissue. Conway et al. [122] reported that for fresh-cut apple *L. monocytogenes* grew in decayed areas infected by *Glomerella cingulata* but populations decreased in decayed areas infected by *Penicillium expansum*. The pH in tissues infected with *G. cingulata* increased from 4.7 to 7.7, whereas the pH in tissues infected with *P. expansum* decreased from 4.7 to 3.7. Similarly, Riordan et al. [123] found that the population of *E. coli* O157:H7 increased 1 to 3 logs in wounded apple tissue infected with *G. cingulata* but no change in the *E. coli* population was observed in wounded tissues infected with *P. expansum*. The pH in the former increased from 4.1 to 6.8 and the pH in the latter showed no significant increase. Increase in pH in fruit tissue infected with *G. cingulata* can therefore promote the growth of human pathogens.

Contrary to the positive effects described above, a number of studies have shown that growth of *L. monocytogenes* on potato slices [124], spinach [42], and endive [23] can be negatively affected or suppressed by the presence of fluorescent pseudomonads, possibly in part due to the production of ion-chelating siderophores [25]. As discussed above, production of acids by *P. expansum* in infected tissue also reduced the growth of *L. monocytogenes* significantly [122,123]. In fact, fluorescent pseudomonads antagonistic to foodborne pathogens can be found commonly on the surfaces of fresh produce and sprouting seeds [23,25,48]. Elimination of spoilage microorganisms such as PF pseudomonads from fresh produce may prolong the shelf life of fresh produce but at the same time may generate a less competitive environment for human pathogens to proliferate to an infectious dosage level. Although not supported by experimental data, it has been suggested [5,11] that an increase in the incidence of the association of foodborne disease outbreak with fresh produce may be in part due to the increase in postharvest treatments for elimination of indigenous microflora on fresh produce.

16.7 POSTHARVEST TREATMENTS OF FRESH PRODUCE AND THEIR EFFECTS ON PF PSEUDOMONADS

After harvest, fruits and vegetables are usually subjected to cleaning and decontamination treatments to remove soil, spoilage microorganisms, and occasional human pathogens. Due to a sharp increase in the association of fresh produce with disease outbreaks during the past two decades [11], extensive research efforts have been made to develop effective treatment methods for enhancing the microbiological safety of fresh and fresh-cut produce [125]. Primary focuses of these studies were to eliminate human pathogens presumably present sporadically at extremely low levels on the surfaces of fresh produce [125]. A number of physical, chemical, and biological intervention technologies [126], previously developed for elimination of

spoilage bacteria and human pathogens on animal food products, have been modified and tested for their efficacy against harmful microorganisms on fresh and fresh-cut produce.

16.7.1 Irradiation

The use of ionizing irradiation (e.g., γ-rays from ^{60}Co or ^{137}Cs, accelerated electrons, or x rays) on raw fruits and vegetables has become a potential means of extending shelf life and inactivating pathogenic microorganisms on fresh produce [127]. Irradiation is measured in grays (Gy) or kilograys (kGy) to indicate a dose of irradiation energy required to kill an organism. Complex life forms with large genomic DNAs are in general more sensitive to the lethal effect of irradiation than simpler organisms with small genomes. The lethal irradiation dose for humans, insects, bacteria, and viruses has been estimated to be 0.004, 0.1, 1.5 to 4.5, and 10 to 45 kGy, respectively [128]. Spoilage of fresh produce caused by *Erwinia* or PF pseudomonads can be suppressed by irradiation at dose levels of 1 to 3 kGy without adversely affecting the sensory qualities of fruits and vegetables [127]. For fresh produce that is more sensitive to irradiation treatments, an even lower dose (0.5 kGy) can be applied, usually in combination with other treatments such as chlorination, heat, or modified atmosphere, to inactivate insect pests [129], spoilage bacteria [130], or human pathogens [131].

16.7.2 Ozone

Ozone has been approved in the U.S. as generally recognized as safe (GRAS) for treatment of bottled water but has not yet been permitted for use as a disinfectant in fresh produce processing [132]. Ozonated water at the concentration of 20 ppm is lethal to most of the bacteria pathogenic to humans or plants such as *P. aeruginosa* [133]. Exposure of *P. fluorescens* to 2.5 ppm of ozone for 40 sec reduces the population of this spoilage bacterium by 5 to 6 logs [134]. Use of ozone to disinfect fruits and vegetables including lettuce has been reported [135]. Moor et al. [136] found that ozone is in general more effective against Gram-negative than against Gram-positive bacteria and is ideal as a terminal disinfectant for food processing because of the lack of odor and residue. Application of ozone is being actively tested for its potential to improve the safety of fresh fruits and vegetables [137].

16.7.3 Chlorine

Chlorine-based sanitizers including elemental chlorine, sodium hypochlorite (NaOCl), calcium hypochlorite (CaOCl), and chlorine dioxide are commonly used disinfectants in washing, spray, and flume waters in fresh produce processing plants. At concentrations of 50 to 200 ppm with a contact time of 1 to 2 min, chlorine is effective in removing over 99% of human pathogens and spoilage bacteria including PF pseudomonads on raw fruits and vegetables [138]. The antimicrobial activity of chlorine is pH-dependent and mainly due to the formation of hydrochlorous acid (HOCl) when dissolved in water. As the pH of the solution is reduced, the equilibrium

is in favor of the formation of HOCl. Fruit and vegetable tissue components can neutralize chlorine, making it inactive against microorganisms. Therefore, the pH and active chlorine content in chlorinated water should be monitored regularly to ensure the maximal antimicrobial effect of chlorine treatment.

16.7.4 Hydrogen Peroxide

H_2O_2 is classified as GRAS for use in food processing as a bleaching agent, oxidizing and reducing agent, and antimicrobial agent. Although it has not been approved for use in the fresh produce industry, the efficacy of H_2O_2 in improving the microbiological quality and extending the shelf life of minimally processed fruits and vegetables has been investigated [139]. H_2O_2 vapor treatments delayed or diminished the severity of bacterial soft rot in fresh-cut cucumber, green bell pepper, and zucchini but had no effect on the spoilage of fresh-cut broccoli, carrot, cauliflower, celery, or fresh strawberry. Similar treatments are able to delay the spoilage of mushrooms caused by *P. tolaasii* but also induce browning in the mushrooms. Dipping fresh-cut zucchini, cantaloupe, or cucumber in an H_2O_2 solution reduced the load of fluorescent pseudomonads by 90% and was similar in effectiveness to chlorine treatment [139]. The presence of H_2O_2 residues in some treated commodities and the adverse effect of treatments on produce color and flavor are the two concerns that require further investigation.

16.7.5 Organic Acids

Organic acids including lactic acid and acetic acid (AA) have been approved for disinfection of beef, lamb, pork, and poultry carcasses. The application of organic acids to the surfaces of fresh produce for the purpose of reducing the populations of pathogenic and spoilage bacteria including PF pseudomonads has been investigated. Gastrointestinal human pathogens such as *Salmonella* and *E. coli* O157:H7 are approximately 10 to 50 times more resistant to AA treatment than plant-associated bacteria such as *Erwinia* or PF pseudomonads. The minimal concentration of AA required to kill 90% of *Salmonella*, *Erwinia*, and *P. fluorescens* within 5 min was estimated to be 2.4, 0.3, and 0.06%, respectively [Liao, unpublished]. Following exposure to AA, a very large proportion of *Erwinia* or PF pseudomonads become injured and are more susceptible to the action of other antimicrobial substances. A combination of AA and H_2O_2 was the most effective treatment against *Salmonella* and possibly spoilage bacteria among five sanitizer treatments examined.

16.7.6 Modified Atmosphere

The use of modified atmospheres (MA) on fresh produce packaged in polymeric film products has a significant effect on the microflora and quality of fresh produce. While small amounts of CO_2 stimulate the growth of many organisms [140], high concentrations of CO_2 (> 3%) inhibit the growth of most organisms, including PF pseudomonads [141–143]. *Pseudomonas* spp. as a group are in general more sensitive to CO_2 than native bacteria found on meat products such as *Proteus*, *Bacillus*, and *Micrococcus*. Thus, MA packaging provides an effective means to extend the

shelf life of produce and to reduce the proliferation of spoilage pseudomonads [20]. The increase in the concentration of CO_2 inside the packaging pouches usually leads to a decrease in the *Pseudomonas* population but often leads to a drastic increase in the population of lactic acid bacteria, which is thought to play a role in spoilage of fresh produce [24].

16.8 CONCLUSION

PF pseudomonads consisting of *P. fluorescens* and *P. viridiflava* are the cause of a large proportion of postharvest rot of fresh fruits and vegetables. They are commonly found on the surfaces of fresh produce and constitute a major component of resident microflora on potato tubers and leafy vegetables. The ability of these pseudomonads to cause spoilage (often in the form of soft rot) results from their ability to produce a variety of depolymerases including pectinases (primarily PL), proteases (Prt), cellulases, and lipases. Unlike multiple PL isozymes produced by *Erwinia*, a single alkaline PL is produced by most if not all PL pseudomonads. The conclusion that a single alkaline PL is the sole or principal pectinase required for induction of soft rot is based on the results from a series of experiments including enzyme purification, isoelectric-focusing electrophoresis, transposon mutagenesis, gene cloning, and complementation studies. Two genes regulating the production and/or secretion of PL, Prt, the exopolysaccharides (alginate and levan), and fluorescent iron-chelating siderophores have been identified. These two genes, designated *gacS* and *gacA*, are members of the two-component regulatory gene family and are predicted to encode a sensory protein for receiving the external or internal signals (GacS) and an activator protein for mediating the synthesis and secretion of the aforementioned extracellular compounds (GacA). The presence of calcium is absolutely required by *P. fluorescens* and *P. viridiflava* to produce and excrete PL and Prt and is also required for catalytic activity of both enzymes. Application of ion chelators such as EDTA and organic acids such as acetic acid and citric acid thus becomes a possible approach for reducing the soft rot caused by PF pseudomonads. The potential of using irradiation, chemical sanitization, and modified atmospheres as well as biological control agents to reduce the population of spoilage and pathogenic bacteria on fresh produce needs to be further investigated.

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